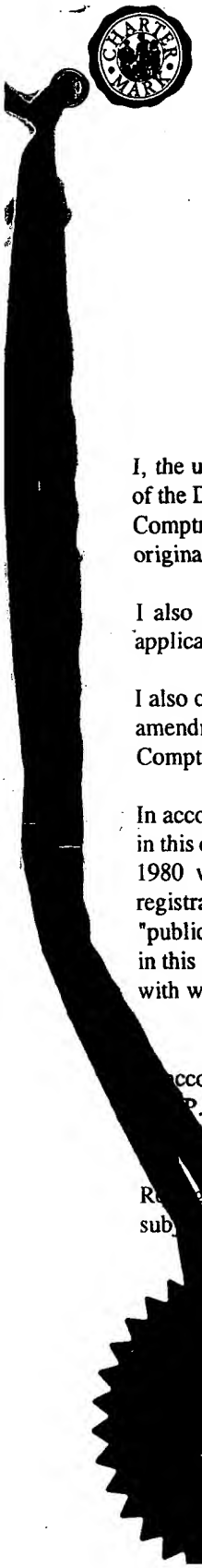

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1. Your reference

2. Patent application number *(The Patent Office will fill in)* 9719834.5 — 18 SEP 1997

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ECLAGEN LIMITED
AURIS BUSINESS CENTRE
23 ST MACHAR DRIVE
ABERDEEN AB2 4RY

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation INCORP. IN SCOTLAND 6628203006

SECTION 30(1)(a) 1977 ACT

APPLICATION FILED 22.09.97

4. Title of the invention

LIGAND-DIRECTED SCREENING FOR BINDING PROTEINS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* ~~As above~~

Kilburn & Strode
20 Red Lion Street
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Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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Number of earlier application	Date of filing *(day / month / year)*
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Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

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LIGAND-DIRECTED SCREENING FOR BINDING PROTEINS

The present invention relates to new screening methods using ligands to direct the binding of proteins encoded by DNA libraries to the vicinity of the ligand binding receptor. In addition, the invention relates to using ligand-directed binding of library proteins for the subsequent isolation of library proteins which bind to the ligand binding receptor.

In order to determine the target receptor protein for a particular ligand molecule, it is feasible to use the ligand for the selective isolation of the target receptor prior to subsequent analysis of the target and it's molecular identification. However, where the affinity of binding by the ligand to it's receptor is low or the ligand and/or receptor are in low abundance, it can be very difficult to isolate the receptor using the ligand and commonly candidate receptors have to be tested by, for example competition assays, in order to determine the identity of the receptor. Furthermore, for the subsequent creation of molecules which block ligand binding (for example, receptor antagonists) or molecules which substitute for the binding of ligand (e.g. agonists), a purified or enriched receptor preparation must be tested with a range of candidate binding molecules (e.g. antibodies) for isolation of molecules which bind at the ligand binding site. Clearly, where the receptor is not available in a purified or enriched form or where the receptor identity is not known, isolation of receptor binding molecules is very difficult. New tools are therefore required for the efficient isolation of target receptors and for the isolation of molecules which bind specifically at the ligand-binding site on the receptor which might act, for example, as agonists, antagonists or simple targeting molecules.

The present invention is based on the principle of using a ligand, which binds to a proteinaceous receptor, to label other protein-complexes binding to the receptor immediately adjacent to the ligand. On this basis, these adjacently binding proteins can then be used as binding agents to isolate the receptor and also, in some cases, to isolate protein molecules adjacent to the receptor. Furthermore, these adjacently binding proteins can subsequently be rebound to the receptor along with other proteins including proteins which bind at the ligand binding site such that the adjacently binding proteins can then label other protein-complexes binding immediately adjacent to themselves including proteins which bind at the ligand binding site. In this manner, one or more protein-complexes which bind at the ligand binding site can be labelled and subsequently isolated and tested for useful properties such as receptor blocking, agonism or antagonism of the receptor. In particular, the invention is useful for the labelling and isolation of molecules on a cell surface and, in particular, on whole tissues. In particular, the invention applies to large DNA libraries encoding protein molecules which can be screened in such a way as to enable the replication of DNA molecules encoding protein molecules of interest, especially those labelled in the method of the current invention.

In the description of the present invention, the term receptor refers to a proteinaceous moiety to which a ligand can bind. The term ligand refers to a molecule, usually proteinaceous, which bind to the receptor. The term binding proteins refers to proteins encoded by a DNA library. Protein-complexes refers to the complex of

binding protein and nucleic acid whereby, upon binding to a target molecule, individual genes encoding the binding proteins can be recovered such that the binding protein can be regenerated. The label refers to a molecule which is released by the labelling moiety and which can combine or react with protein-complexes via the label-receptor. The labelling moiety refers to the carrier or source of the label referring commonly to an enzyme which can subsequently catalyse the release of the label. The label-receptor refers to the site on the protein-complex where the label binds.

A preferred feature of the present invention is the use of liposomes as the labelling moiety to provide the label which labels the protein-complexes binding immediately adjacent to the ligand whereby the ligand is or can become associated with liposomes which are made to release their encapsulated contents in the vicinity of the ligand. This provides a great versatility in the use of different labels in the method of the present invention. Other labelling systems such as the enzyme activation system described in US5196306 can also be used within the method of the present invention.

A preferred feature of the present invention is the use of protein-complexes each comprising a mRNA molecule, one or more ribosomes and one or more translated proteins. In such a complex, the protein(s) act as the binding protein(s) whereby, upon successful binding, the protein-complex can be recovered and the mRNA replicated usually by conversion to cDNA, commonly using PCR, and subsequent transcription (by virtue of a transcriptional promotor introduced by synthetic DNA) or cloning into replicable vectors. Another preferred feature of the present invention is a replicable nucleic acid gene library from which can be derived protein-complexes. Commonly, genes from this library will encode antibody variable regions (commonly in the form of single-chain Fv's, or scFvs) although libraries of other proteins can also be used especially libraries encoding conserved internal protein frameworks with variations in the external amino acids and protein structures/shapes. It is a requirement of the present invention that the protein-complex can be labelled by the label in such a way that labelled protein-complexes can be preferentially isolated away from unlabelled protein-complexes.

In the preferred embodiment of the present invention, there is provided a proteinaceous ligand which binds to a previously uncharacterised receptor on the surface of a cell or tissue section. The ligand is either combined directly before binding with bacterial phospholipase C (PLC) by chemical conjugation or combined indirectly usually after binding with, for example, an anti-ligand antibody itself conjugated to PLC. Following binding of the ligand to the cell, the protein-complexes derived from the DNA library are added to the cell/tissue to allow for the protein components to bind to the cell/tissue and excess non-binders are removed by washing. This effectively leaves a cell or tissue with a multitude of surface bound protein-complexes including some complexes in close vicinity to the bound ligand including some actually bound to the uncharacterised receptor for the ligand. In the next step, a preparation of liposomes are added which encapsulate a label. Upon contact of the liposomes with the cell or tissue with surface bound protein-complexes, the only lysis of the liposomes will occur through contact with PLC which hydrolyses lipid head-groups to destabilise the liposome at the point of PLC contact. This leads to the leakage of the label from the liposome. Whilst the label can diffuse away from the

site of PLC contact, it is a principle of the present invention that a suitable label can efficiently react with a suitable label-receptor only in the vicinity of the PLC. Such a label is streptavidin which can bind tightly to biotin molecules associated with the protein-complex, usually on the mRNA component by virtue of an annealed synthetic oligonucleotide. An alternative label is the HIV tat protein or a peptide fragment thereof which can bind to a hairpin-loop (TAR) on suitable mRNA molecules. Having reacted with the adjacent protein-complexes, excess liposomes and label are washed away from the cell/tissue and the labelled protein-complexes are recovered. Recovery can be effected usually using an immobilised label or derivative of the label (e.g. biotin) or an antibody which specifically binds to the label (e.g. anti-biotin, anti-tat). Various measures can be employed to remove the labelled protein-complexes from the cell/tissue surface without dissociating the protein-complexes although the genetic component of the protein-complexes can also be removed preferentially by, for example, addition of EDTA to dissociate mRNA/ribosome/protein complexes. The recovered labelled protein-complexes or genetic component of the protein-complexes can then be subjected to nucleic acid amplification in order to expand the numbers of nucleic acid molecules and to facilitate subsequent re-cloning of DNA into a suitable replicable vector or, where transcription/translation is used, to permit subsequent transcription by virtue of a promotor introduced via synthetic DNA used for gene amplification. Thus, genes encoding one or more binding proteins which bind to the cell/tissue adjacent to the ligand can therefore be isolated to provide an abundant source of binding protein to facilitate in the molecular isolation of the receptor and also possibly in the molecular isolation of molecules in the vicinity of the receptor which may include molecules which are activated by the receptor in the transmission of a signal to the interior of the cell.

In addition to facilitating the molecular isolation of the receptor, the binding proteins isolated by the method of the present invention can themselves be used effectively as ligands for a subsequent round of cell/tissue binding whereby the original binding proteins are themselves conjugated or attached to one or more PLC molecules. Following binding of these binding protein-PLC conjugates to the cell/tissue at the receptor site, a library of gene-derived protein-complexes are then added to bind to the cell/tissue. From a suitable library, this would provide binding proteins which bind both to the natural ligand binding site of the receptor itself and also to other proteins in the vicinity of the receptor. Upon liposome addition and lysis upon contact with PLC molecules, new protein-complexes would be labelled with labels such as streptavidin or tat allowing their subsequent isolation or enrichment. By retrieval of the genes encoding the binding proteins in these labelled binding protein-complexes, a number of additional tools for receptor and adjacent molecule isolation may be provided. However, in some cases more desirable, one or more of the new labelled protein-complexes might bind to the natural ligand binding site and therefore might provide new binding proteins which might block binding of the natural ligand or which might substitute for the natural ligand in activating the receptor or which might induce an antagonistic response at the receptor through receptor-induced down regulation of activities which are normally up-regulated by the natural ligand. In some cases, individual binding proteins might provide candidate pharmaceutical molecules.

It will be recognised by those skilled in the art that the method of the present invention will usually be conducted using a DNA library comprising a collection of genes encoding different protein types, some of which might bind to the target receptor.

It will be recognised by those skilled in the art that the ligand molecule can be any molecule, proteinaceous or non-proteinaceous in nature, to which can be attached either directly or indirectly, a labelling moiety capable of releasing the label to attach to the protein-complex.

It will be recognised by those skilled in the art that the binding protein could be based upon several molecular types. Whilst the protein type will commonly be antibodies encoded by antibody variable regions (commonly in the form of single-chain Fv's, or scFvs), other proteins can also be used especially those with conserved internal protein frameworks whereby variations in the external amino acids and protein structures/shapes will generate a library of different protein molecules. Some of the shapes adopted by such non-antibody protein molecules may be more compatible to binding to the target receptor than antibodies themselves which are suitable for binding to other protein molecules with relatively flat binding faces but less suitable to binding to protein molecules with complex shapes such as concave shapes to accommodate large proteinaceous ligands.

It will be recognised by those skilled in the art that the protein-complex could be one of several types whereby the binding protein is linked either directly or indirectly to the nucleic acid sequence encoding it. In the preferred embodiment, the protein-complex is a complex of mRNA/ribosome/protein which is formed by translation of mRNA under conditions designed to optimise yield of complexes in preference to translated proteins which have terminated and become free from association with ribosome sand therefore mRNA. In other embodiments, the protein-complex will comprise bacteriophage particles derived from gene library which display the binding protein on the phage surface. Alternatively, the protein-complex will comprise bacterial particles which display the binding protein derived from gene library on the bacterial surface. Other embodiments will include lacI fusion proteins comprising fusions of lacI and the binding protein whereby lacI binds to the lac operator on a plasmid encoding the binding protein and other viral protein display systems including baculovirus and retroviruses.

It will be recognised by those skilled in the art that the label can be one of many molecules which can bind to other proteinaceous or nucleic acid molecules in a protein-complex in the vicinity of the labelling moiety which releases the label. It will also be recognised that the corresponding label receptor can be one of several molecules matched to the label. Suitable labels (and label receptors) will include streptavidin (biotin), tat (TAR hairpin loop on mRNA), signal recognition particle (SRP) (bacterial leader sequence on protein), antibody (antigen), RNA binding molecules (mRNA), specific protein binding molecules (binding sequence on protein), F pilus (bacteriophage F receptor) and nickel (histidine "tag" on protein).

It will be recognised by those skilled in the art that the labelling moiety will commonly be an enzyme which causes the release of the label. Such labelling

moieties will include PLC (for liposome, red blood cell, or other cell lysis), horseradish peroxidase (for biotinyl tyramide) and bacterial enzyme beta-galactosidase (for lysis of glycoliposomes). It will also be recognised that, where the labelling moiety is a porin or membrane molecular channel, this may also cause the release of the label directly.

It will be recognised by those skilled in the art that other liposome lytic mechanisms could be used within the context of the present invention as an alternative to enzyme lysis. For example, it may be possible to attach whole liposomes to ligands either directly, for example by encapsulating the ligand with a lipid tail into the liposome membrane in such manner as to still permit ligand binding to a receptor, or indirectly, for example by using liposomes impregnated with surface antibodies or other binding proteins which target the liposome to the ligand. Under such circumstances, liposome lysis could be simple achieved by detergent lysis or heat or any other conventional ways of achieving liposome lysis.

It will be recognised by those skilled in the art that the method of the present invention could be used to directly label receptors and other molecules in the vicinity of receptors rather than to label protein-complexes. For example, using a PLC-conjugated ligand and liposomes containing streptavidin, the whole cell/tissue could be chemically biotinylated using, for example, biotin-NHS ester and then the PLC-ligand and subsequent liposome lysis would deposit strptavidin molecules specifically in the vicinity of the ligand including on the receptor which would therefore become labelled with streptavidin providing a basis for molecular separation using, for example, using immobilised biotin.

The following example illustrates the invention but should not be considered to limit the scope of the invention:

Example 1: Isolation of Epidermal Growth Factor Receptor
The monoclonal antibody 340 (PCT00443) was used as a ligand for the epidermal growth factor receptor (EGFR). 1mg antibody 340 was conjugated to 10mg PLC (Sigma, Poole, UK; #P4039) using Sulpho-MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester) (Pierce,Chester, U.K.; #22312) as cross-linking reagent. Conjugation was performed using the manufacturers recommended protocol. Reagents were desalted and purified post-conjugation using G25 Sephadex columns (Pharmacia, Uppsala Sweden; #17-0851-01), and conjugation verified by SDS polyacrylamide gel electrophroresis and Coomasie Blue staining according to standard protocols (in *Antibodies, A Laboratory Manual* eds Harlow E. & Lane D. Cold Spring Harbour Laboratory Press 1988, NY, USA).

Methods for producing monoclonal and polyclonal anti-EGFR antibodies were standard methods essentially as described in *Antibodies, A Laboratory Manual* ibid. Mouse monoclonal antibodies reactive to EGFR were obtained using the human breast carcinoma MDA-MB468 as immunogen for BALB/c mice. Primary and secondary injections were both with $5x10^5$ cells intraperitoneally 3 weeks apart followed another week by 4 injections over 2 days into the tail vein. After a further 5 days, spleens were removed and fused to SP2/0 mouse myeloma cells according the procedure of Kennet, R H; in *Methods Enzymol.*, vol 58 (1978) p345-359. Pooled

hybridomas were tested for EGFR binding activity as described by Modjtahedi, H et al., Br J Cancer, vol 67 (1993) p247-253 and expanded to $5x10^7$ cells for mRNA isolation using a Fast Track mRNA isolation kit (InVitrogen, NV Leek, Netherlands). mRNA was then processed using a Pharmacia Recombinant Phage Antibody System kit (Pharmacia, Milton Keynes, UK) according to the manufacturer's instructions to produce a library of 10^7 cfu bacteriophage from the pCANTAB5 vector.

For transcription, the vector pET-5 was used (Promega, Southampton, UK) to provide a promoter, translational start site and terminator from the bacteriophage T7 gene 10. Initially, a 3' spacer sequence was cloned into this vector based on the glycine-rich linkers of gene III of filamentous phage M13 which was generated by PCR using the following primers;

m13f1: GGC TTT AAT GAA GAT CCA TTC
m13f2: CCG TAT GGA TCC GGC TTT AAT GAG GAT CCA TTC
m13r1: CTG TAG CGC GTT TTC ATC GGC
m13r2: CCG TAT AGA TCT CTG TAG CGC GTT TTC ATC GGC

Two sets of PCR reactions were performed using primer combination m13f1 and m13r2 or with m13f2 and m13r1. These two sets of reactions generated two populations of products, one with a 5' *BamH*I restriction site and one with a 3' *Bgl*II restriction site. The restriction sites were included to facilitate the construction of multimers of the 30 amino acid linker. The *BamH*I containing PCR products were digested and phosphatased and then ligated with the *Bgl*II digested PCR products. In this way multimers ligated only 5' to 3' were formed. A 900 bp multimer was selected and ligated to a self annealed synthetic oligonucleotides encoding the HIV transactivation response element (TAR) as follows;

TAR1: 5'GATCAGCCAGATTTGAGCAGC
TAR2: 5'GATCGCTGCTCAAATCTGGCT3' (5' end dephosphorylated)

The fragment was repurified and cloned into the *Bam*HI site of pET-5 to yield the plasmid pET-5III with the gene III/TAR insert, an upstream *Bam*HI site but no downstream site.

Inserts in pCANTAB5 were amplified using the following primers;
forward: 5'CCG TAT GGA TCC GCG GCC CAG CCG GCC ATG GC3'.
reverse: 5'CCG TAT GGA TCC CCC GTG ATG GTG ATG ATG ATG3'.
PCR amplification reactions were performed using Boehringer Expand High Fidelity PCR system. Reaction conditions for amplification of DNA fragments were 1x Expand HF buffer, 2.5 mM $MgCl_2$, 4 mM of each dNTP, 2.5 units of polymerase, 10 ng template DNA and 30 pmol of primer DNA. Reactions were incubated in a Perkin Elmer thermal cycler 480 using the following programme: 92°C for 5 min, 67°C for 5 min, 72 °C for 1 min, followed by 30 cycles of 92°C for 1 min, 67°C for 1 min and 72°C for 1 min. The resultant 795 bp fragment was then purified using Wizard PCR purification columns (Promega) and cloned into the modified vector pET-5III at the *Bam*HI site by digesting the PCR product and vector with *Bam*HI.

In vitro transcription of 10ug of the mixed library DNA was performed using the RiboMAX large scale RNA production system(Promega) according to the manufacturers instructions. The resultant mRNA was purified using PolyATtract system (Promega).

In vitro translation was performed in an *E. coli* S-30 system as described by Chen and Zubay (ibid) modified as described by Hanes and Pluckthun (ibid) and supplemented with HIV tat 37-72 peptide (Naryshkin *et al.*, Biochemistry **36** (1997), p3496-3505). The translation was stopped after 10 minutes and the mixture centrifuged as described by Hanes and Pluckthun (ibid).

For the liposomal-tat preparation, liposomal vesicles were produced by mixing the lipids L-phosphatidylcholine and oleoyl-palmitoyl cholesterol. Tat 37-72 peptide at a concentration of 100mM in 10mM Tris pH8.0 was added to 10mg of a mixture of 1.5:1 L-alpha-phophatidylcholine and cholesterol (Sigma Poole U.K; #13906). Vesicles were formed following repeated cycles of vigorous mixing and allowing the solution to stand at room temperature. The volume of the liposomal solution was increased by addition of 10mM Tris 10mM pH8.0 and liposomes purified from unincorporated components by gel filtration through a G25 Sephadex column (Pharmacia, Uppsala Sweden; #17-0851-01) with borate buffered saline (BBS; 0.2M sodium metaborate, 7.5g/l NaCl, 1.8g/l $CaCl_2.2H_20$, pH adjusted to 7.0 with boric acid). Integrity of the eluted liposomes was assessed by microscopy where intact liposomes were compared to control preparations lysed by treatment with a solution of 1% (v/v) NP-40 (Pierce, Chester, U.K.; #28324). Liposomes were diluted in BBS to give a final concentration of 10mM tat 37-72 peptide.

Anti-tat antibody was made using the tat peptide 37-72 which was conjugated via its N-terminal cysteine residue to KLH using MBS according to *Antibodies, A Laboratory Manual* ibid. 10ug of the conjugate was used to immunise Balb/c mice as above and serum was collected and used at 1:100 dilution in the further experiments.

791T/36 cells (Doran M et al., Br J Cancer, vol 62 (1990) p500) were grown up and harvested in trypsin/EDTA and washed twice in RPMI1640 medium before being resuspended at $5x10^5$ cells/ml. 100ul of cell suspension was then mixed with 100ul of 100ug/ml of 340-PLC conjugate in PBS supplemented with 5% (w/v) bovine serum albumin (BSA) (Sigma, #A7906, Poole, UK) and incubated at 37c for 1 hour. Cells were then centrifuged and washed twice in ice-cold phosphate buffered saline (PBS) containing 0.1% (v/w) BSA and resuspended in 100ul PBS. The translation reaction derived from an original 1ug of library DNA was then added in 100ul PBS and incubated at 37c for a further 2 hours. Cells were again centrifuged, washed twice in PBS containing 0.1% (v/w) BSA and resuspended in 100ul of this buffer. 100ul prepared liposome suspension was added and incubated for 1 hour at 37^0C. Cells were subsequently washed for 3 x 5 minutes in PBS containing 0.1% (v/w) BSA.

Tat-bound mRNA was recovered by dissociation of mRNA in EDTA buffer as described by Hanes and Pluckthun (ibid) followed by passage through a column comprising polyclonal anti-tat antibody on protein A beads prepared according to *Antibodies, A Laboratory Manual* ibid. Washing and dissociation of retained

ribosome complexes, isolation of mRNA, reverse-transcription PCR and repeated transcription-translation with a final round of labelling using 35S methionine label at 50uCi/ml (Amersham International, Amersham, UK) were as described by Hanes and Pluckthun (ibid). After 3 rounds of ribosome display on 791T cells, the PCR products were transcribed and translated with 35S methionine and the mixture was then applied to Immulon 2 96-well microtitre plates (Dynatech, Chantilly, VA, USA) coated with an EGFR antigen preparation from human placenta made using an antibody 340 immunoaffinity column (PCT00443) and coated onto the plates in coating buffer according to the manufacturer's protocol. Approximately 1ug of translated protein was added per well either with 0.1ug BSA alone or with 1ug 340 antibody or 5ug polyclonal anti-EGFR (as above). Plates were incubated for 1 hour, washed 5 times with PBS containing 0.1% Tween 20 (Sigma) and bound protein was eluted with 0.1M triethylamine and counted in a scintillation counter. The results, as shown in table 1, indicate specific binding of translated protein derived from triplicate mRNA/ribosome/protein complexes eluted from 791T cells using anti-tat columns but not from triplicate complexes eluted and processed without anti-tat enrichment or triplicate complexes directly from the DNA library. Furthermore, the lack of inhibition of binding by 340 antibody which comprised the PLC-labelled ligand in the experiment indicates that binding is specific to epitopes on EGFR other than that of 340.

cpm - ^{35}S

Origin of Translation Complex	Translation Mix alone	Translation Mix +340 Mab	Translation Mix + anti-791T
791T selected	2050	1750	340
791T/anti-tat selected	21210	18420	3930
Library only	130	100	110

Table 1: